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TERMINAL REPORT

MICROBIOLOGY OF ANAEROBIC SLUDGE

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Summary

Studies on the microbiology of anaerobic sludge disclosed that non-methanogenic strictly anaerobic bacteria comprised one of the most numerous groups of organisms present. These previously unreported organisms are mainly un-named Bacteroides species. One of the commonest organisms in our study was closely related to Bacteroides ruminicola subsp. brevis on the basis of physiological similarities and growth requirements. Morphologically, it was more like subsp. ruminicola. Euryoxic bacteria constitute only a minor fraction of the normal bacterial flora but can become predominant in very short time if fermentable carbohydrates are present. This finding emphasizes the necessity of using unenriched fermentations for studying the sludge digestion process. Lipids are the main substrates responsible for generation of acetate in the unaltered normal sludge fermentation. Other substrates such as carbohydrates and proteins are not as acetogenic. Control of the fermentation on a practical scale must take into account the role of lipids as substrates. Organisms responsible for lipid decomposition are apparently slow-growing and are washed out of the digestor at short retention times. Recycling of the bacteria would enable the operator to shorten retention time without a loss in efficiency of decomposition.

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An understanding of the sludge-methane fermentation can be achieved by a study of the various types of bacteria which participate in the breakdown of complex organic compounds to end-products which serve as substrates for the ultimate formation of methane. This phase of the sludge fermentation (the so-called "acid-producing" phase) has been almost completely overlooked although it is recognized as an important unexplored aspect of this fermentation. The present investigation is an ecological study of the physiological types of bacteria which are responsible for degradation of organic compounds to methanogenic end-products, primarily acetic acid. Certain criteria have been established (1) for studies of this type and appear suitable for application to the sludge habitat. These include: 1) evaluation of the total microbial activity (by some measurement of the overall metabolism); 2) enumeration of the most significant types of bacteria; 3) establishment of the relative contributions of the various bacteria to the total fermentation. Progress has been made toward the determination of these objectives although much remains to be examined.

1) Evaluation of the total microbial activity. The magnitude of the sludge fermentation is determined by the overall rate of methane formation. It was found in previous investigations (3,8) that methane is produced primarily from acetic acid (70%) with propionate and butyrate accounting for most of the remaining 30%. Determination of the relative rates of formation of acetic, propionic, and butyric acids was made after

feeding the digester with raw sludge. Figure 1 shows that acetic acid has the fastest rate and is present at highest concentration. The increase in acetic acid is accompanied by an increase in both propionic and butyric acids. The concentration of any of these acids is dependent upon its turnover rate, i.e., its rate of formation relative to its rate of utilization. Since the concentration increases after feeding, its rate of formation must be greater than its rate of degradation. At steady state the concentration is constant since the rate of formation is equal to the rate of utilization. The turnover numbers at steady state concentrations of acetic, propionic and butyric acids have been previously determined and the relative contribution to methane production of these acids estimated (acetic acid, 70%; propionic and butyric acids, 30%; see above). The starting substrates for these acids were presumed to be carbohydrates, proteins and amino acids, and fats. Jeris and McCarty reported that when carbohydrates, fats and amino acids were metabolized, 70% of each substrate was converted to methane and CO_2 by way of acetic acid. Although these results are of interest with respect to the possible pathway of metabolism, no information is presented on the actual contribution of each type of substrate to the normal fermentation. In addition, these studies were made with enriched systems which were acclimated to the test substrate. Our studies are based on the assumption that metabolism of test substrates should be examined without alteration of the resident bacterial population by prior enrichment.

The necessary substrates for the production of organic acids must be initially present in the raw sludge. The nature of the substrates and

their contribution to the acetate pool was not examined until recently (5,6). Since organic acids increase in concentration immediately after feeding raw sludge, their rate of formation must be greater than their rate of utilization. Short-term incubation of digesting sludge in the presence of substrates such as carbohydrates, fats, and proteins should also lead to such an accumulation of acid end-products. The relative contribution to the acetic pool of each class of substrates may be estimated by determining their maximum rates of acetate production.

Short-term incubation of glucose (0.5% final concentration) as a model carbohydrate test substrate led to some unexpected results. Figure 2 shows the results obtained upon addition of glucose, cellobiose, and sucrose to digesting sludge. The sludge control represents the endogenous rate of metabolism; gas production was measured manometrically as an index of fermentation. All of the substrates were metabolized, but vigorous gas production occurred only after a 10-11 hr lag and was followed by a 3-hr period during which gas was produced exponentially, finally levelling off after 20 hrs. Such a lag is expected if enzymes were induced in resident organisms present at high concentrations or if organisms initially present at low concentration grew during this incubation period. Glucose was chosen for further investigations since all three substrates followed similar kinetics of utilization.

Figure 3 shows a comparison of gas production from glucose with that from a raw sludge substrate which contains other carbon sources in addition to carbohydrates. Gas production from the raw sludge substrate occurred at a steady and initially higher rate than for glucose, indicating that some substrates other than glucose were being fermented. However, the glucose vessel again exhibited an exponential increase in gas production

after a 10-hr. lag. The subsequent rate as well as total gas production far exceeded the control. The raw sludge substrate contains a mixture of various organic compounds which might not be as energetic as glucose alone. The increased rate of glucose utilization probably reflects the availability of energy for growth and enrichment of glucose-metabolizing organisms.

The production of individual volatile acids and glucose disappearance were investigated with respect to gas production. Figure 4 (upper graph) depicts the rates of production of acetic acid, propionic acid, and ethanol; all increase at the same time as gas production, which is shown in the lower graph. Acetic acid and ethanol were produced in higher concentrations than propionic acid. Other organic acids, such as valeric, isovaleric, and butyric which are separated by gas chromatography were not detected in these samples. Analyses were not made for formic and lactic acids. The bottom graph shows that disappearance of glucose, as detected by the glucostat method, corresponds to the period of rapid gas formation.

To determine whether growth of glucose-using organisms accounted for this gas production, viable counts were made under both aerobic and anaerobic conditions at various times during incubation of the sludge with glucose. The total anaerobic counts showed an increase in numbers of 3-10 fold and do not disclose any information concerning growth of organisms initially present at low concentrations. However, examination of aerobic counts showed a thousand-fold increase in numbers of euryoxic organisms which could account for the sudden increase in gas formation

from glucose. These data are shown graphically in Figure 5. Euryoxic organisms show an exponential increase in numbers after the typical 10-hr lag period whereas total anaerobic counts increased only by a factor of 10.

Anaerobic counts of acid-producing colonies were detected in sludge-glucose media with a brom-thymol blue-calcium carbonate indicator. Colonies were selectively counted and isolated at the beginning and at the peak of gas formation from the highest dilution exhibiting growth.

Results of aerobic counts obtained with conventional pour-plates and anaerobic counts obtained in this indicator medium are depicted in Figure 6. It is apparent that the anaerobic counts of acid-producing organisms substantiated the findings of an increase in organisms initially present at low dilutions. Acid-producing colonies increased 100-1000 fold in the anaerobic medium and corresponded to a similar increase in aerobic counts.

Since there was no apparent initial increase in gas production in the presence of these carbohydrates, uniformly labeled (UL) glucose was added and the rate of $C^{14}O_2$ was measured to determine the rate of glucose utilization at the moment of addition. Samples were killed by acidification with H_2SO_4 at 10-min intervals, the $C^{14}O_2$ trapped with hyamine and radioactivity detected by scintillation counter. Figure 7 shows these data for sludge at 0 time and after 12 hr incubation in the presence of glucose. Glucose uptake was calculated under the assumption that 2 moles of $C^{14}O_2$ were produced per mole of glucose dissimilated. These data established that glucose is used immediately by normal sludge; the rates

of utilization were 1.3×10^{-3} and 3.4×10^{-2} uM/ml/min for the 0-time and 12-hr samples respectively. The increase in the rate of glucose metabolism is 20-fold although we have already seen the increase in acid-producing and euryoxic colonies is 100-1000 fold. These findings indicated that the initial utilization of glucose is not accounted for by acid-producing colonies alone.

Several organisms were isolated from anaerobic roll tubes inoculated with glucose-fermenting sludge. Two of the isolates were strict anaerobes and produced acetate and propionate. A third strict anaerobe produced only propionate. The remaining isolates were euryoxic and produced acetate and ethanol. These end-products corresponded to those produced by the mixed sludge population.

Unfortunately, the above isolates were difficult to transfer and were subsequently lost. A second series of organisms was isolated and characterized. They were obtained from the following dilutions:

A. From a dilution of normal sludge which had never been fed glucose.

Group 1. Isolation of colonies from the highest dilution showing growth (10^{-7} dilution).

Group 2. Isolation from the highest dilution of indicator medium showing acid-producing colonies (10^{-3} dilution).

B. From a dilution of sludge incubated with glucose for 14 hr.

Group 3. Acid-producing colonies from the highest dilution of indicator medium (10^{-7}).

A summary of the organisms isolated is shown in Table 1.

Number and Designation of Organisms Isolated			
	Strict Anaerobes	Euryoxic	Euryoxic, Growing Better Anaerobically
Group 1	8: S2, S3, S4, S5, S6, S7, S9, S10	0	0
Group 2	4: S2-6, R1, R2 R5	7: R8, R10, S2-4	1: R2
Group 3	3: C4; G2-6 G2-7	9: G1, G3, G5, G7 G8, G9, G2-1 G2-4, G2-9	6: G2, G10; G2-3, G2-5 G2-8, G2-10

Organisms from Group 1 were randomly isolated from the highest dilution showing growth (10^{-7}). These isolates were all strict anaerobes in confirmation of our earlier findings ( ); none of them produced sufficient organic acid end-products to dissolve the $CaCO_3$ indicator. This further substantiates the observation that glucose-using, acid-producing organisms were not present in the highest dilutions at 0 time. Table 2 gives some of the characteristics for differentiating these anaerobic bacteria. Included in this table are organisms of both the euryoxic (but growing better anaerobically) and strictly anaerobic type. The classification of these organisms is not yet complete; all of them appear to fit within the Bacteroides and have not been further identified. They do not conform to the characteristics of previously described Bacteroides species. Most acid-producing isolates formed acetate and ethanol from glucose.

Examination of Table 3 shows the euryoxic isolates which fall into three main categories. Group A are all organisms of the Providence group; Group B are of the Paracolobactrum type; and Group C are of the Escherichia coli type.

These findings show that "acclimatization" of digesters to artificial substrates may be preceeded by an enrichment for organisms not present in significant numbers in the normal digester. Euryoxic bacteria were shown to be responsible for the rapid degradation of glucose in the present investigations. Such organisms were found at 10^3 to 10^6/ml in unenriched sludge whereas the most numerous organisms are anaerobes (4) which occur at 10^8 to 10^9/ml. Thus, previous reports pertaining to the formation of methane from various synthetic substrates are subject to this shift in population. It should be further noted that in the case of glucose-enriched systems a marked change in the bacterial flora occurs within a 24-hr period and the population of organisms at this time is by no means representative of the unenriched system, which contains slow-growing strictly anaerobic organisms. Many previous reports on glucose-enriched systems are predicated on the incorrect assumption that only slow-growing strict anaerobes are significant (2). Our studies show that euryoxic organisms are capable of growing in glucose-fed sludge at generation times less than 20 min and are the predominant organisms in glucose-fed sludge after only 14 hr incubation. Thus, in all of our studies, none of the samples were ever enriched with pure substrates.

The chief substrates responsible for acetogenesis have not been reported in spite of the fact that acetic acid is the single most important source of methane. Various fractions of sludge were

were tested at twice the normal concentrations to accentuate the acetate production. A raw sludge substrate control was also tested at twice its normal concentration. The results (Figure 10, upper graph) indicated that the lipid fraction contained the substrates responsible for the increase in acetate after addition of raw sludge. The lower graph compares acetate production when two and four times the normal amount of lipid served as substrates. The similarity of these data indicated that even the lower quantity of lipid exceeded a limiting concentration.

The production of acetate from chemically pure substrates was also examined; they were tested at a concentration of 0.2% (w/v). The upper graph in Figure 11 indicates that palmitic acid and glycerol were acetogenic. However, the results observed for glycerol were not repeatable, and in subsequent experiments glycerol was not acetogenic. The rate of formation of acetate from palmitic acid was similar to that from an equivalent amount of lipid extract of raw sludge. Acetate was not produced from octanoic acid. This result was contradictory because palmitic and octanoic acids are both saturated straight-chain fatty acids and should be metabolized in a similar manner. Tripalmitate was also not acetogenic. This result suggested that lipases responsible for hydrolyzing palmitic acid from glycerol may not be present in sludge.

Since palmitate was highly acetogenic, its metabolism was investigated further by using palmitate-U-^{14}C. Stock solutions of labeled palmitate and unlabeled palmitate were prepared by dispersing the palmitate in 0.2% Tween 80 and sonicating for 30 minutes at 60 C.

About 0.2 μc/ml palmitate-U-^{14}C and 0.1% cold palmitate were added to test tubes containing 10 ml of digesting sludge. After incubation for 6 hr at 35 C the samples were killed with 3% metaphosphoric acid. Zero-time samples were killed prior to addition of the palmitate. Control digesters without labeled palmitate were also included. Formate, acetate, propionate, and butyrate were separated by celite chromatography, and the activities were determined by liquid scintillation spectroscopy. The amount of label added was determined by measuring the specific activity of the 0-time sample; 1 μl of supernate was added to 2 ml of absolute ethyl alcohol and 15 ml of scintillation fluid. The results of this study disclosed that palmitate was converted to acetate. Under conditions in which methanogenesis was inhibited, palmitate was converted to butyrate. The fact that butyrate was the major end-product of palmitate dissimilation under these conditions was not in accordance with the β-oxidation pathway of metabolism. Butyrate may have resulted from secondary reactions of acetate. Such reactions might serve to remove hydrogen produced during dissimilation of palmitate since the formation of methane was inhibited in these experiments.

Although carbohydrates, lipids, and proteins are more or less equally distributed in raw sludge, lipids are decomposed to a greater extent. In addition, the lipid substrates in raw sludge are dissimilated at a faster rate than other substrates. Addition of raw sludge results in increased rates of gas and volatile acids production which can be attributed to the fermentation of lipid. Other compounds in raw sludge contribute to the slower fermentation rates

of endogenous sludge.

Thus, the rates of fermentation and degree of dissimilation of various substrates differ. These differences are a reflection of the types of bacteria resident in the particular sludge under study. At any given moment, the population of bacteria is dependent upon a number of factors such as pH, temperature, substrates, oxidation-reduction, and retention time. Demonstration of the importance of lipids in this study is a good example of the influence of retention times, assuming the other variables were held constant. A long retention time permits development of organisms with long generation times; a short retention time would eliminate such organisms. The short retention times reported by McCarty (5) and O'Rourke (6) resulted in sludge which could not decompose lipid, probably because lipid decomposers were washed out of the system by the short retention time. The normal sludge retention time in treatment plants is long enough to permit growth of lipid decomposers since lipid dissimilation occurred at rapid rates in all such samples tested. Lipids are, in fact, degraded at higher rates than other substrates examined. Surprisingly, carbohydrates were not rapidly dissimilated.

Organisms which promote rapid dissimilation of carbohydrates were present only in low numbers. However, in the presence of excess substrates, they responded by an energetically wasteful conversion of the carbohydrates to acid end-product and because of their short generation time (20 minutes), became the predominant organisms in a very short period. The average number of the non-methanogenic organisms reported by previous investigators is higher than that for methane

organisms. The significance of these reported figures may be due to the greater immediate dependence of the methane organisms on other bacteria for formation of hydrogen which is oxidized as an energy source during the reduction of carbon dioxide tomethane. Thus, the heterogeneous bacterial population present in sludge can be regarded as a population of opportunists, eachof which can gain predominance because of favorable selective pressures. It is the balanced activity of these organisms which makes the sludge fermentation a practical treatment process for disposal of organic material via methane formation.

2. Enumeration of the most significant organisms

. The predominant organisms in the sludge fermentation are strictly anaerobic non-methanogenic bacteria. Euryoxic bacteria are present in smaller numbers. While not surprising, this finding was not previously reported; the only information existing on the evaluation of anaerobic bacteria from this system dealt with cellulose-decomposing or methane-producing bacteria.

Several of our isolates have been studied in detail and the characteristics are recorded in Table 4. These organisms were sent to Dr. W.E.C. Moore's Anaerobic Laboratory for substantiation of characteristics and similar results were obtained. Several appear to be species and/or strains of previously undescribed Bacteroides. We have been able to classify 3 strains of one of the most commonly occurring colony types.

The anaerobic methods and media reported by Mah and Sussman (4) were employed in the pure culture studies. Inorganic salts were

included in all media except litmus milk and blood agar. Fermentation media were prepared by addition of the test substrate at a final concentration of 1% in sludge supernatant broth media (33% v/v clarified sludge supernatant fluid plus inorganic salt solution). Gas production medium was tubed in 10-ml quantities and consisted of sludge supernatant agar plus 1% final concentration of glucose. In some cases, the synthetic medium of Caldwell and Bryant was used after modification by addition of the inorganic salts solution reported by Smith (7). Gelatin liquifying medium contained the final percentage concentrations of: Difco nutrient broth, 0.8; Difco gelatin, 12.0; resazurin, 3.0×10^{-5}; $NaHCO_3$, 0.4; cysteine-HCl, 0.05; Na_2S, 0.025; glucose, 0.1; inorganic salts. Nitrate reduction medium contained the following concentrations in percent: Difco nitrate broth, 0.9; glucose, 0.1; Difco purified agar, 0.1; cysteine-HCl, 0.05; $NaHCO_3$, 0.4; inorganic salts.

The three isolates were termed C-1, C-2, and C-3. Each produced colonies of similar appearance; in roll tubes, they appeared white with slightly textured surfaces and some iridescence. The edges of the colony were usually smooth but sometimes became irregular. Surface colonies were convex, and deep colonies were lenticular.

All three isolates were gram negative rods with round ends and ranged in size 0.8 to 1.0 μ wide and 3.0 to 4.7 μ long. The organisms occurred predominantly in singles or pairs, but some long chains were usually observed. All three isolates had capsules; no spores or motility were ever observed.

The results of these studies are shown in Tables 5 and 6. In some cases, pure cultures of organisms which produced known reactions in the medium tested were used as positive controls. Whenever appropriate, negative uninoculated controls were also kept.

The nutritional studies reported in Table 7 show that hemin was not a growth requirement for these isolates. Although the present strains resemble Bacteroides ruminicola subsp. brevis in its hemin requirement as well as its fermentation products, they are not morphologically typical of this subspecies. Instead, they resemble B. ruminicola subsp. ruminicola more closely since both tend to be rods rather than cocci. It is not surprising that such intergrading types are found in nature because of the artificial taxonomic criteria necessarily chosen for any delimitation of bacterial species.

3. Establishment of the relative contribution of the various bacteria to the total fermentation. Information on this aspect of our study is dependent on solution of some of the problems in the previous two categories. Determination of the key substrates in this fermentation will be an important first step towards determination of the responsible organisms and the relative importance of the organisms participating.

4. Although most of the methane produced comes from the dissimilation of acetate, propionate, and butyrate, a complete analysis of the source of methane must include the minor intermediates as well. Formic acid is an important end-product of anaerobic metabolism and although it is a minor component of sludge, determination of its role in methanogenesis is of some importance.

The contribution of each intermediate to methane formation can be assessed by determining its turnover number (8). During the fermentation, any given intermediate is maintained at steady-state concentrations because of a balance between its formation and its conversion to methane. If a small amount of radioactively labelled intermediate is introduced into the steady-state pool, its rate of disappearance will be an index of the turnover number at known pool concentrations. The amount of label added should not be high enough to alter the pool size.

The kinetics of this reaction follows a simple first order reaction, $\frac{dx}{dt} = kx$, and is treated exactly the same as the growth equation in which the variables are separated and integrated between the limits of initial and final radioactivity at given times t and t_0. The result is an equation for a straight line in which k represents the slope and ln(x at t) is the Y intercept. k is thus determined by a log plot of the decrease in radioactivity versus time and has the units of reciprocal time. The product of k times the pool size, x, gives the turnover number expressed in our experiments as μM/ml/min.

With these objectives in mind, the role of formate during sludge methanogenesis was examined. A small amount of C^{14}-labelled sodium formate of high specific activity was added to a series of rubber-stoppered test tubes containing 5-ml of sludge. Addition was accomplished by injection with syringe and hypodermic needle through the rubber stopper while the sample was being vigorously agitated on a cyclomixer. At appropriate time intervals, the samples were killed by injection of acid or alkali, chromatographed to separate formate, and the

activity measured by scintillation counter. The pool size was determined enzymatically with formyl tetrahydrofolic acid synthetase reduction of THF at 350 mμ. Total and external pool size was determined directly; internal pool was obtained by difference. Rates of methane formation were estimated by manometry. The results of such an experiment are shown in Fig. 12.

The loss of formate shown by these data indicate that formate disappears very rapidly. The same results were obtained when the experiment was repeated on three separate occasions. The rate constant calculated from this graph is 41 min^{-1}, and the turnover number is 0.71 uM/ml/min. Of this, the bulk must be due to an equilibrium reaction. The rapid rate observed could not be due to simple turnover of the formate to methane since it would mean a rate of methanogenesis many times in excess of the actual rate determined by manometry. It seems more likely that this loss resulted from exchange reactions with the CO_2 pool as indicated by the disappearance of formate from solution with a corresponding appearance of radioactive CO_2 in the gas phase (Fig. 13). Furthermore, the formate pool remains permanently labelled at low activity--probably due to an equilbrium between the labelled gas and formate. This is supported by the finding that the final specific activities of the calculated CO_2 and formate pools are very nearly the same.

Tritiated sodium formate was used with the idea of circumventing this exchange phenomenon. Samples were killed at 15 and 30 second intervals by injection of saturated NaOH to a final concentration of 1.5N. Results are given in Fig. 14. Determination of total activity

showed that the tritium exchanged rapidly with water. The total count of the whole sludge remained unchanged throughout the sampling. Fractionation of the sludge showed that formate decreased rapidly in activity. The loss from formate must be by exchange with water and not some other non-volatile compound since virtually 100% of the radioactivity remaining after evaporation and drying of the whole sludge sample could be accounted for in the formate. All of the label can be accounted for at any time by analysis of the formate and water. This interchange reaction apparently occurs by a different mechanism than that for CO_2 since formate hydrogen is not in equilibrium with molecular hydrogen but rather with hydrogen in solution. The presence of this reaction precludes a direct reduction of formate to methane without a prior interchange of hydrogen. In other word, the formate hydrogen is not obligatorily bound to the carbon as is the case with the methyl group of acetic acid during methanogenesis.

In order to estimate the maximum rate of methane which can be formed from formate, the exchange reaction should be minimized. If the formate pool size were increased, the formation of enzyme-formate complex would be favored over that of enzyme-CO_2 or CH_4 rather than interchange. Addition of labelled bicarbonate to an unlabelled formate pool increased to a final concentration of 20 μM/ml showed that incorporation of $C^{14}O_2$ into the formate pool was inhibited. Uptake under these conditions was approximately 16% of the expected rate. If this is the case, then addition of labelled formate at a concentration of 20 uM/ml should result in minimizing the dilution due to exchange with CO_2 and formation of labelled CH_4 would more nearly reflect its formation from formate. Assuming that the specific activity

of the methane is the same as formate, the maximum possible amount of methane formed was estimated. On this basis, the cpm of C^{14} appearing in methane was converted to μM and the results plotted in the next graph (Fig. 15). The rate of methanogenesis was calculated to be 3×10^{-3} μM/ml/min of sludge. This amounts to approximately 4% of the total methane produced. It is understood that this estimate is the maximum contribution assuming reduction of the formate carbon without mixing. Actually, availability of formate hydrogen under these conditions leads to an increase of two times the normal rate of methanogenesis.

The finding that formate may account for 4% of the total methane produced during sludge digestion is in accordance with the rates of methanogenesis from other sludge intermediates. Previous results have shown that acetate alone contributes as much as 73% of the total methane and more recent findings show that propionic acid accounts for 13% and butyric acid for 8%. Thus, of the total methane formed during the sludge fermentation, 98% has been accounted for.

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	Gram Stain	Motility	Cellobiose	Esculin Hyd.	Glucose	Glycerin	Lactate	Starch Hyd.	Gas Prod.
C2; C10; S3; R1; R2; R6	GN Rods	+	-	- non hyd.	+	-	-	+ hyd.	+
C4; S2-3	GN Rods	-	+	± no acid hyd.	+	-	-	+ hyd.	-
S4	GN Rods	-	-	- hyd.	+	-	-	+ hyd.	-
S7	GN cocco-bacilli	-	-	- hyd.	+ −	-	-	- non hyd	-
S2; S5; S6; S8;S9; S10	GN Rods	-	+	± no acid hyd.	+	± no acid	-	- non hyd	+++
S2-6		-	-	- hyd.	+	-	-	± no acid hyd.	-
C2-6		-	-	± no acid hyd.	+	-	-	+ hyd.	-
C2-7			-	- hyd.	+	-	-	- hyd.	-
C2-3; C2-5 C2-8; C2-10			+	± no acid hyd.	+	± no acid	-	+ hyd.	++

TABLE

	Group A G1, G3, G5, G7, G8, G9	Group B R8, R10	Group C S2-4; S2-5; S2-7; S2-8; S2-9; C2-1; C2-4; C2-9
Gram stain	GN Rods	GN Rods	GN Rods
Growth on:			
Blood agar	+++	+++	+++
Plate count agar	+++	+++	+++
Mac conkey	+++	+++	+++
Citrate	+	+	-
Gelatin	-	-	-
H_2S	-	-	-
Indol	-	+	+
Litmus milk	Acid & peptonization after 10 days	Acid & peptonization after 10 days	
Methyl red	+	+	+
Motility	+	+	+
Phenylalanine	+	-	-
Urea	-	-	-
V. P.	-	-	-
Adonitol	-(±)	-	+G
Cellobiose	+	+G	-
Dulcitol	-	-	-
Esculin	-	-	(+G)
Galactose	+	+G	+G
Glucose	+	+G	+G
Glycerin	+	+G	(+G)
Inositol	-(±)	-	-
Lactose	+ after 2 weeks	+ after 10 days	+G
Maltose	+	+G	+G
Monnose	+	+G	+G
Mannitol	-	+G	+G
Raffinose	-(±)	-	-
Rhamnose	-	+G	+G
Salicin	+ after 2 weeks	+ after 10 days	(+G)
Sorbitol	-(±)	+G	+G
Sucrose	+	-	-
Starch	+	-	-
Trehalose	+	+G	+G

- = negative
+ = growth
G = Gas
( )= Delayed

TABLE 3

2/

	5-1		5-2A		5-3		5-4	5-6A		5-7		5-9		5-11		6-1	
PYG	+++ 5.1	+	+++ 5.75	+++	+ 6.4	+	+++	+++ 5.8	+	+ 6.2	+	++ 6.1	-	+ 6.2	-	++ 6.4	-
Adonitol	+ 6.03		++ 6.78		- 6.4			+ 6.55		± 6.4		= 6.5		-- 6.38		- 6.62	
Amygdalin	+ 5.6		+ 6.05		6.6			+ 6.2		+ 6.15		+ 6.5		+ 6.38		+ 5.75	
Arabinose	++ 5.5		++ 6.51		+ 6.3			+ 6.1		- 6.2		+ 6.25		± 6.25		- 6.22	
Cellobiose	++ 5.56	+	+++ 6.15	++	± 6.4	±	-	++ 6.3	+	++ 6.3		+ 6.45	±	+ 6.35	=	- 6.35	±
Cellulose	+ 6.35		+ 6.4		++ 6.35			+ 6.4		+ 6.1		+ 6.3		+ 6.39		- 6.3	
Dextrin	+ 5.6		+ 6.08		+ 6.2			+ 6.3		+ 5.85		+ 6.4		+ 6.46		+ 6.0	
Dulcitol	+ 6.31		+++ 6.66		± 6.35			+ 6.4		+ 6.22		+ 6.5		± 6.45		- 6.5	
Erythritol	+ 6.51		++ 6.85		+ 6.55			+ 6.55		+ 6.4		+ 6.3		+ 6.21		+ 6.5	
Esculin	+ 6.1	+	+++ 6.65	+++	+ 6.4	±	±	++ 6.35	+	+ 6.45	+	+ 6.4	±	± 6.41	---	- 6.48	±
Hyd.	++++	+	+++	+	+++	-	-	+++	+	+++	+	+++	+	++++	+	++++	
Fructose	++ 6.03		++ 5.8		++ 6.15			+ 5.9		+ 6.1		+ 6.35		+ 6.34		+ 6.28	
Galactose	++ 5.78		++ 6.1		± 6.15			++ 5.9		+ 5.9		+ 6.3		+ 6.3		+ 6.3	
Glycerol	+ 6.43	+	++ 5.9	++	+ 6.5	+	±	++ 6.7	+	++ 6.6	±	± 6.5	±	+ 6.39	±	- 6.7	
Glycogen	+ 5.62		++ 6.21		+ 6.3			++ 6.4		++ 6.25		+ 6.38		++ 6.19		+ 6.25	
Inositol	+ 6.4		+ 6.51		- 6.25			+ 6.35		++ 6.35		± 6.3		+ 6.13		+ 6.35	
Inulin	++ 6.5		+++ 6.75		+ 6.45			++ 6.3		+ 6.3		+ 6.4		± 6.35		+ 6.7	
Lactate	+ 6.11	+	++ 6.41	++	+ 6.15	±	-	+ 6.35	+	+ 6.2	+	+ 6.0	±	+ 6.0		+ 6.3	±
Lactose	++ 5.75		+++ 6.71		++ 6.4			++ 6.35		- 6.5		+ 6.2		± 6.39		± 6.7	
Maltose	++ 5.72		+++ 5.59		+ 6.3			+ 6.45		++ 6.15		+ 6.15		+ 6.22		+ 6.7	
Mannitol	+ 6.4		++ 5.79		+ 6.42			++ 6.7		+ 6.2		+ 6.25		+ 6.25		-- 6.8	
Mannose	++ 5.75		+++ 6.55		+ 6.35			++ 6.4		+ 6.5		+ 6.2		+ 6.28		- 6.5	
Melezitose	++ 5.8		++ 6.8		+ 6.1			+ 6.05		++ 6.28		+ 6.2		+ 6.31		-- 6.2	
Melibiose	++ 5.82		+++ 6.76		+ 6.3			++ 6.15		- 6.4		+ 6.0		+ 6.3		- 6.55	
Pectin	++ 5.69		++ 5.91		++ 6.25			++ 6.0		+ 6.2		+ 6.2		++ 6.3		- 6.25	
Pyruvate	++ 6.4		+++ 6.71		+ 6.4			++ 6.35		++ 6.2		+ 6.3		++ 6.5		++ 6.22	
Raffinose	+ 5.6		++ 6.7		- 6.3			+ 6.05		+ 6.2		+ 6.3		± 6.42		+ 6.15	
Rhamnose	+ 5.72		++ 6.6		++ 6.35			+++ 6.7		++ 6.4		+ 6.2		+ 6.35		+ 6.6	
Ribose	++ 5.69		++ 5.5		+ 6.35			++ 6.3		+ 6.5		+ 6.15		+ 6.26		+ 6.58	
Salicin	+ 5.8		++ 6.21		- 6.5			++ 6.25		+ 6.3		+ 6.35		+ 6.38		+ 6.5	
Sorbitol	++ 6.7		+ 6.5		+ 6.25			++ 6.7		++ 6.6		+ 6.55		± 6.55		- 6.65	
Sorbose	+ 6.18		+ 6.35		+ 6.25			+ 6.35		+ 6.2		+ 6.15		+ 6.25		+ 6.35	
Starch	+ 5.5	+	++ 5.6	++	+ 6.1	±	±	++ 5.7	±	+ 5.95	+	+ 6.2	±	+ 6.28	±	+ 5.8	±
Hyd.	+	-	+	+	-	-	-	+	-	-	-	+		+		-	-
Sucrose	+ 5.55		+++ 5.8		+ 6.35			++ 6.2		+++ 6.15		+ 6.3		+++ 5.5		+++ 6.1	
Trehalose	+ 5.38		++ 5.86		+ 6.1			++ 6.0		++ 5.7		+ 6.3		+ 6.35		- 6.35	
Xylose	+ 5.91		++ 6.2		- 5.95			++ 6.4		+ 5.9		+ 5.95		+ 6.05		+ 5.95	
Gelatin liq		-		-		-	-		-		-		-		-		-
Gas prod		-		++		-	-		-		-		-		-		-
	Bacteroides		Butyrivibrio					?		Bacteroides		Bacteroides		Bacteroides			

TABLE 4

TABLE 5
RESULTS OF INOCULATION INTO VARIOUS MEDIA

Medium	Positive control	Negative Control	Results for C-1, C-2, C-3
SS broth (1% glucose)	-	Broth (no glucose) Broth (no inoculum)	Turbid with slimy-like sediment. Acids produced-succinic, acetic, some formic
Indicator (0.01% glucose)	-	-	Dark golden colonies with no halo
Nitrate broth	Aerobacter aerogenes	Broth (no inoculum)	Neither nitrite nor N_2 produced
Gas production	-	Medium (no inoculum)	Small amounts produced. Is not CH_4, H_2, H_2S. Colonies only below oxygenated level
Blood agar	Inoculated SS agar	-	No growth
SIM[illegible] Motility Indole H_2S	Proteus vulgaris Escherichia coli Proteus vulgaris	Medium (no inoculum)	Negative Negative Negative
SS broth (1% glucose; 30% CO_2, 70% H_2	SSS broth (1% glucose; 30% CO_2, 70% N_2)	Broth (no inoculum)	Good growth
SS agar (0.01% glucose)	-	-	Minute colonies within 24 hours
Litmus milk	-	Medium (no inoculum)	Soft clot, proteolysis (clearing), acid
Peptone agar	SS agar (0.01% glucose)	Medium (no inoculum)	Scant if any growth. No gas
Gelatin	Staphylococcus aureus	Medium (no inoculum)	Liquefaction

TABLE 6
RESULTS OF FERMENTATION STUDIES*

Substrate	pH	Growth	Final pH	Growth	Final pH	Growth	Final pH
Control-no substrate	7.0	0	7.0	0	7.0	0	7.0
Glucose	6.9	++++	4.7	++++	4.7	++++	4.6
Cellobiose	7.0	++++	4.8	++++	4.7	++++	4.7
Maltose	6.9	++++	4.8	++++	4.8	++++	4.7
Fructose	7.0	++++	4.8	+++	4.8	+++	4.8
Lactose	7.0	++++	4.7	++++	4.7	++++	4.6
Soluble starch**	7.0	++++	4.7	++++	4.7	++++	4.7
Esculin**	6.8	++++	5.5	++++	5.4	+++	5.3
Xylan*** (0.5%)	7.0	-	5.9	-	6.2	-	6.3
Cellulose*** (12% v/v of a 3% suspension)	7.1	-	7.2	-	7.0	-	7.0
Casein hydrolysate (acid)	6.9	0	6.9	0	7.0	0	6.9
Casein hydrolysate (enzymatic)	7.1	0	7.0	0	7.0	0	7.0
Peptone	7.0	+	7.0	+	7.0	+	7.0
Glycerol	7.0	+++	5.1	+++	5.0	+++	4.9
Sodium acetate (0.2%)	7.0	0	7.0	0	7.0	0	7.0
Sodium propionate (0.2%)	7.0	0→+	7.0	0→+	7.0	0	7.0
Butyric acid (0.2%)	6.7	0	6.7	0→+	6.7	0	6.7
Sodium lactate	7.0	0	7.1	0	7.1	0	7.1
Control-Tween 80	7.0	0	7.1	0	7.0	0	7.1
Octanoic acid in	6.8	0	6.8	-	-	-	-
Tween 80 (0.5%)	7.0	-	-	0	7.0	0	7.0
Linoleic acid***	7.1	-	7.1	-	-	-	-
in Tween 80 (0.5%)	7.0	-	-	-	7.0	-	7.0
Tripalmitin*** in Tween 80 (0.25%)	7.0	-	7.1	-	7.1	-	7.0

* In duplicate. Average reported. Substrate concentrations of 1% except where [illegible] turbidity.

** Hydrolyzed by all three strains.

*** Growth not detectable due to turbidity of broth itself.

TABLE 7
NUTRITIONAL STUDIES*

Medium	C-1	C-2	C-3
Control (complete synthetic medium)**	++++	++	++
No hemin	+++	++	+++
No trypticase	+++	++	+++
No yeast extract	+++	+++	+++
No sodium acetate	++++	++	+++
No sodium propionate	++++	++	++++
No butyric acid	++++	++	++
No isobutyric acid	+++	++	++
No n-valeric acid	++++	++	++
No isovaleric acid	++++	+	++
No methyl-n-butyrate	++++	++	+
No NH_4Cl	++++	+++	++
No $MgCl_2$	++++	++	++
No $CaCl_2$	++++	+++	++
No $(NH_4)_6Mo_7O_{24}$	++++	+	++
No $CoCl_2$	++++	++	++
No KH_2PO_4	++++	+++	++
No yeast extract, No trypticase	1/2+	1/2+	1/2+
No Yeast extract, No trypticase, No cysteine	1/2+	1/2+	1/2+
No volatile acids	++++	++	++
Minimal	0	0	0
Minimal plus vitamins	0	0	0
Amino acid A	0-1/2+	0-1/2+	1/2+
Amino acid A plus vitamins	1/2+	1/2+	1/2+
Amino acid B	1/2+	1/2+	1/2+-+
Amino Acid B plus vitamins	1/2+	0-1/2+	1/2+
Minimal plus vitamins plus PAB***	0	0	0
Amino acid A plus vitamins plus PAB***	1/2+	1/2+	1/2+
Amino acid B plus vitamins plus PAB***	1/2+	1/2+-+	1/2+-+

* Performed at least in duplicate. 1% glucose was added as a carbon source in all cases since preliminary studies showed insignificant growth without glucose.

** TThe modified medium of Caldwell and Bryant (6). Except for the omissions noted, all other ingredients were present.*** Biotin and PAB (p-aminoben

*** Biotin and PAB (p-aminobenzoic acid) were selected as vitamin supplements because a strain of bacteria (4) to which these 3 isolates appear closely related required these vitamins. Thiamin and nicotinic acid were added to provide additional possible requirements.

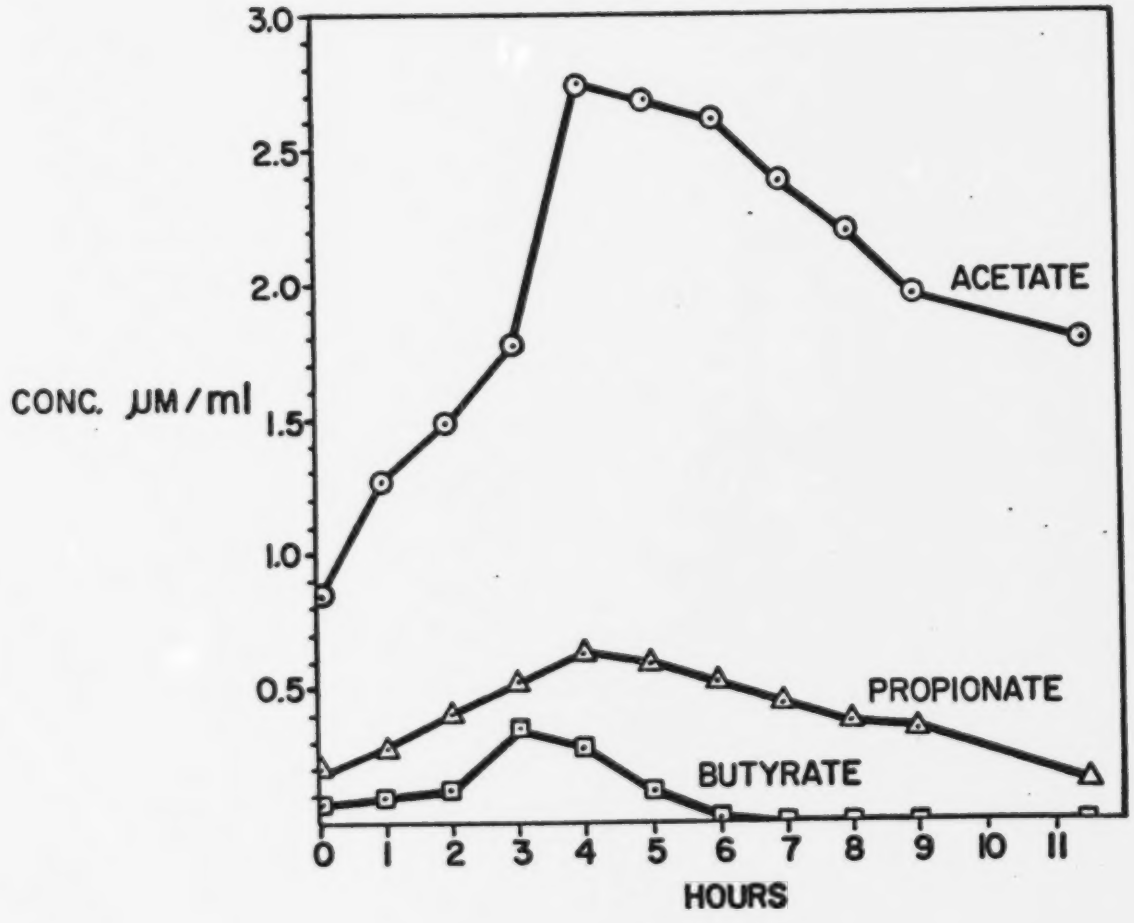
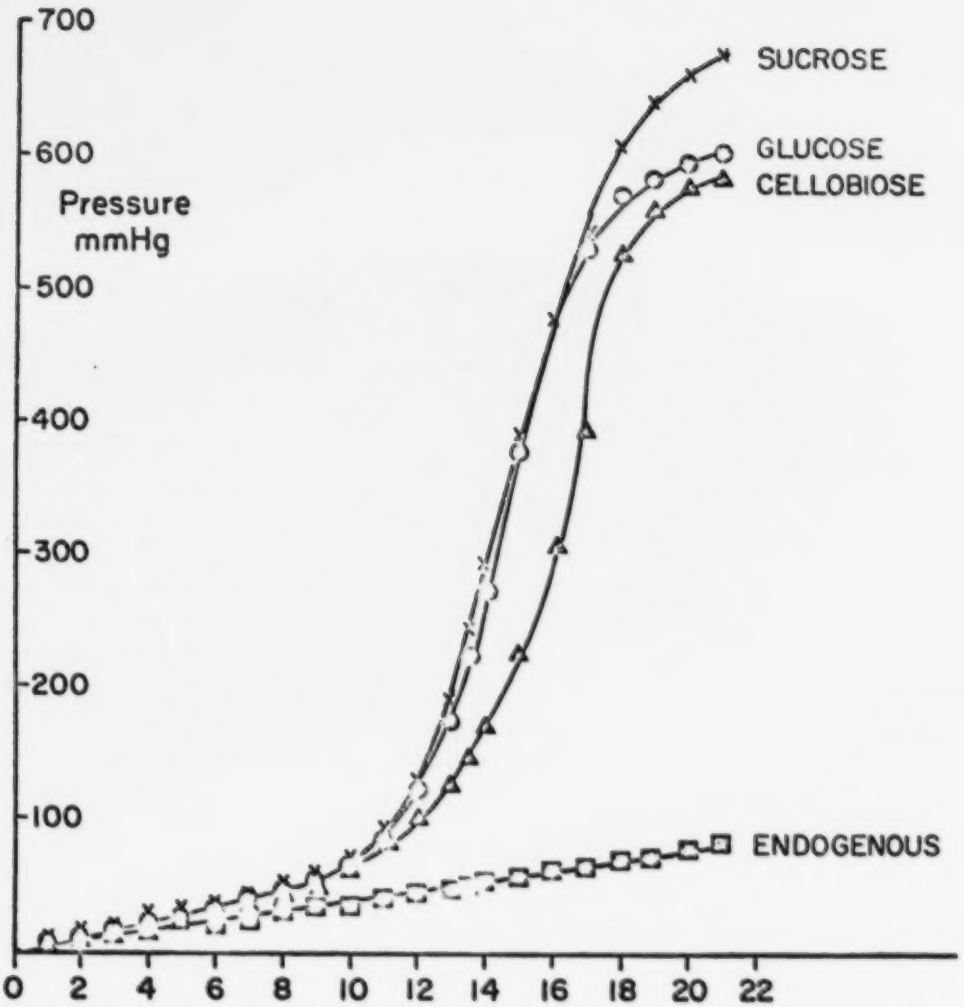
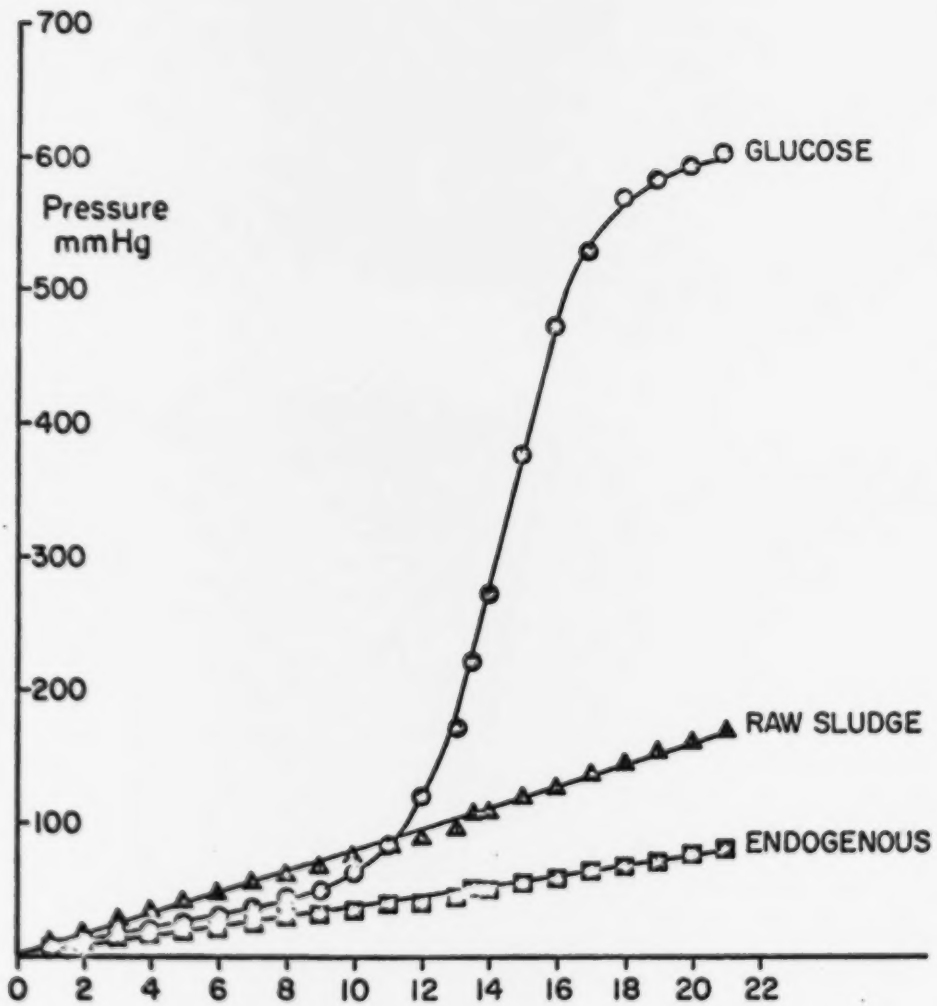

3.0
2.5
2.0
1.5
1.0
0.5
CONC. µM/ml
ACETATE
PROPIONATE
BUTYRATE
0 1 2 3 4 5 6 7 8 9 10 11
HOURS

FIGURE 1



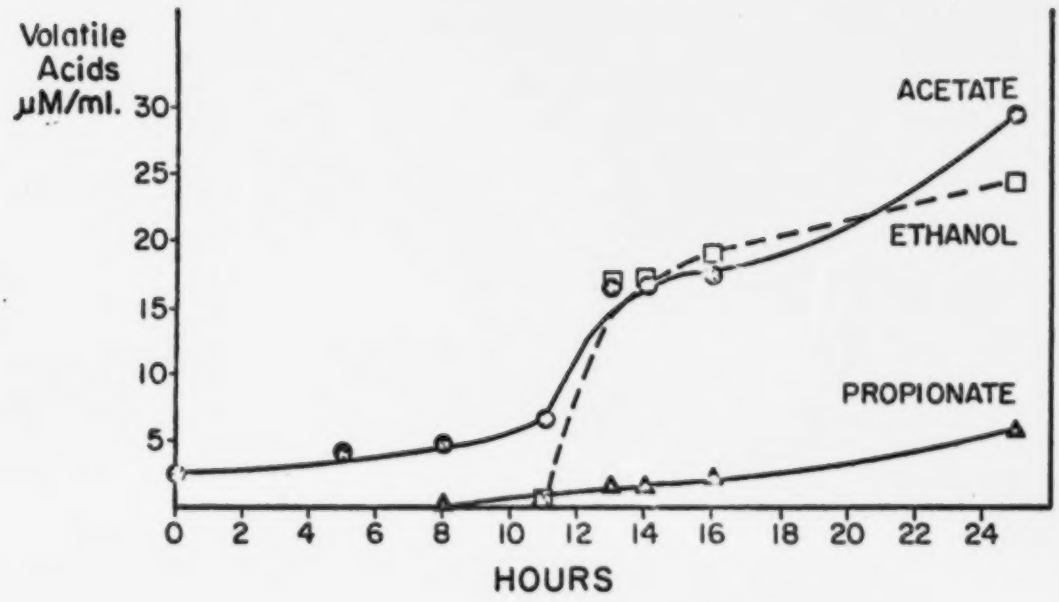
FERMENTATION OF SUGARS BY ANAEROBIC SLUDGE

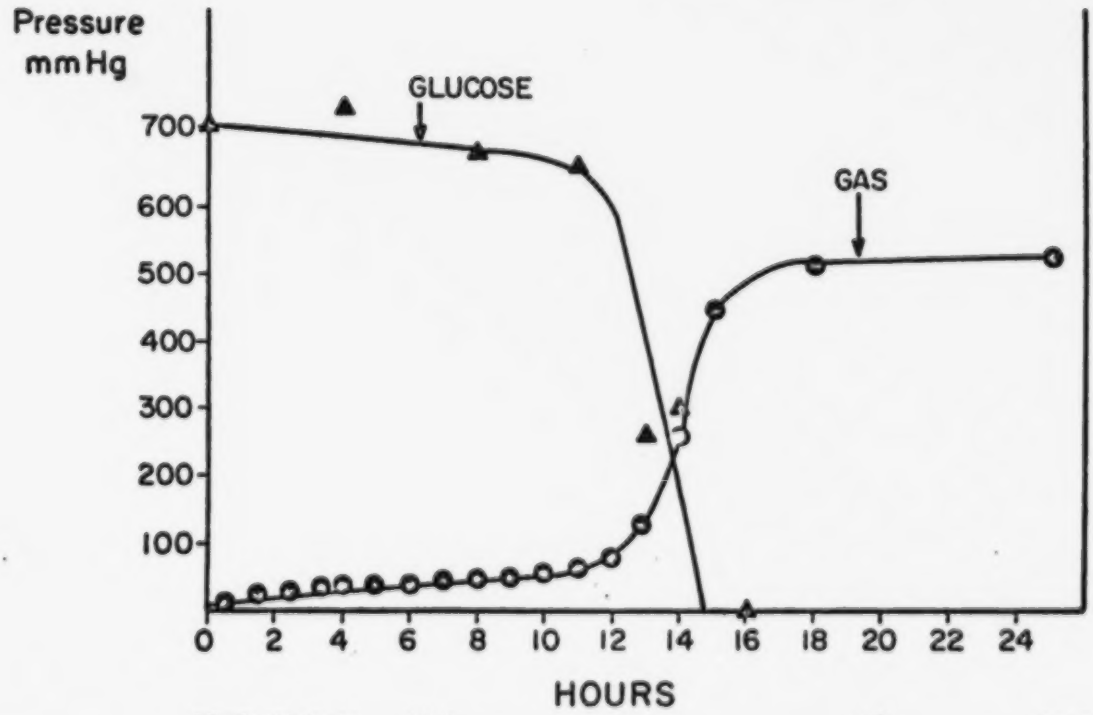
FIGURE 2



FERMENTATION OF GLUCOSE AND RAW SLUDGE

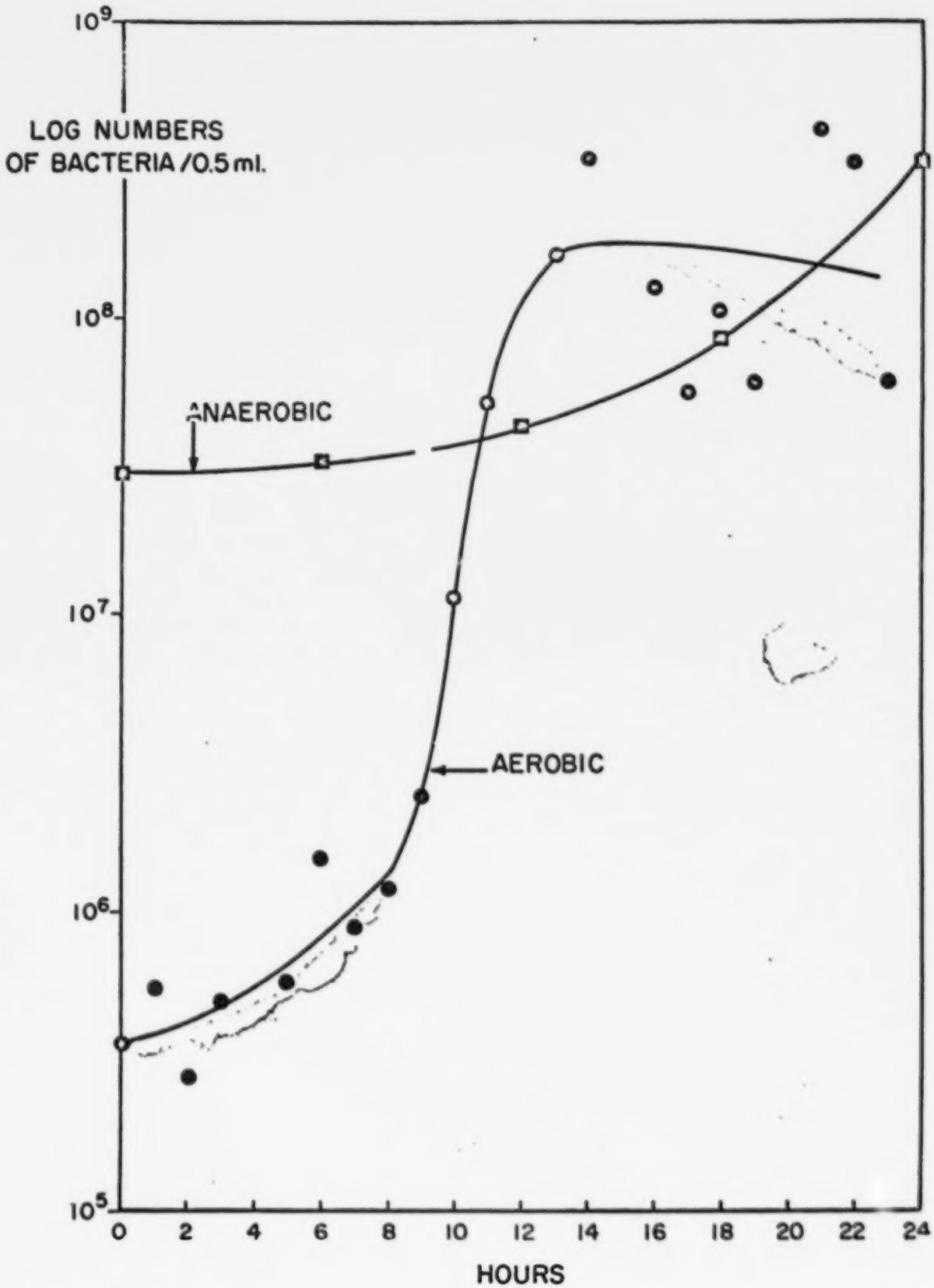
FIGURE 3





GLUCOSE DISAPPEARANCE AND END PRODUCT ANAEROBIC SLUDGE

FIGURE 4

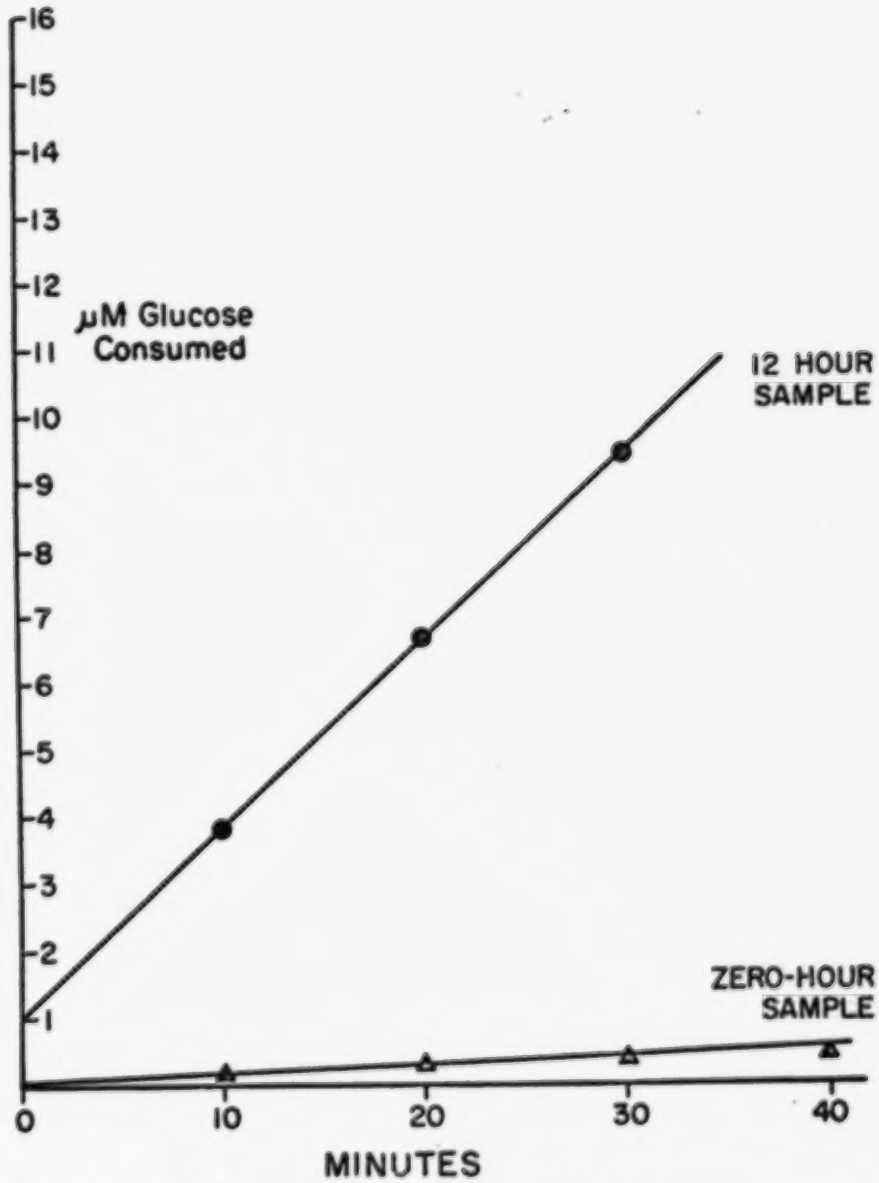


VIABLE COUNTS DURING GLUCOSE FERMENTATION

TOTAL VIABLE AEROBIC AND SELECTIVE ANAEROBIC COUNTS DURING GLUCOSE FERMENTATION BY SLUDGE

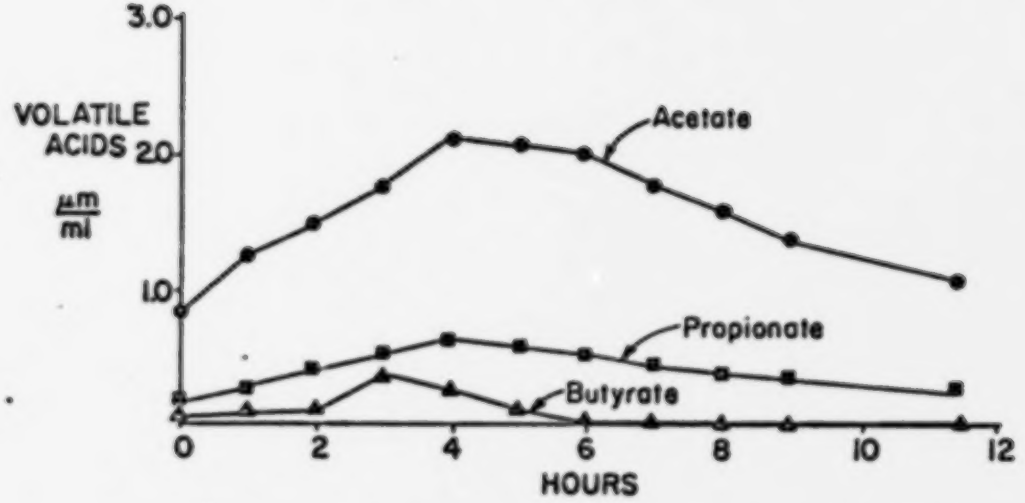
Experiment Number	Time Hours	Counts/ml	
		Selective Anaerobic	Aerobic
3	0	5.0×10^5	---
	24	9.2×10^7	---
4	0	2.0×10^4	---
	14	2.6×10^7	---
5	0	9.9×10^6	6.5×10^6
	10.5	7.4×10^8	9.3×10^8

FIGURE 6



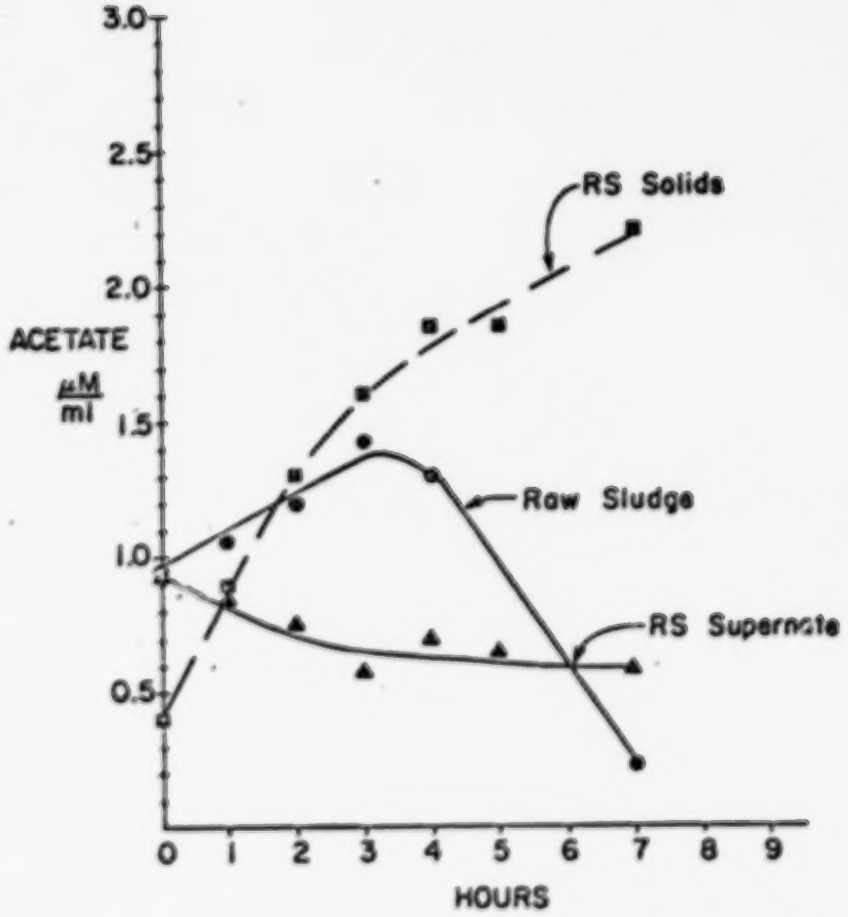
GLUCOSE UPTAKE CALCULATED FROM $C^{14}O_2$ PRODUCTION

FIGURE 7



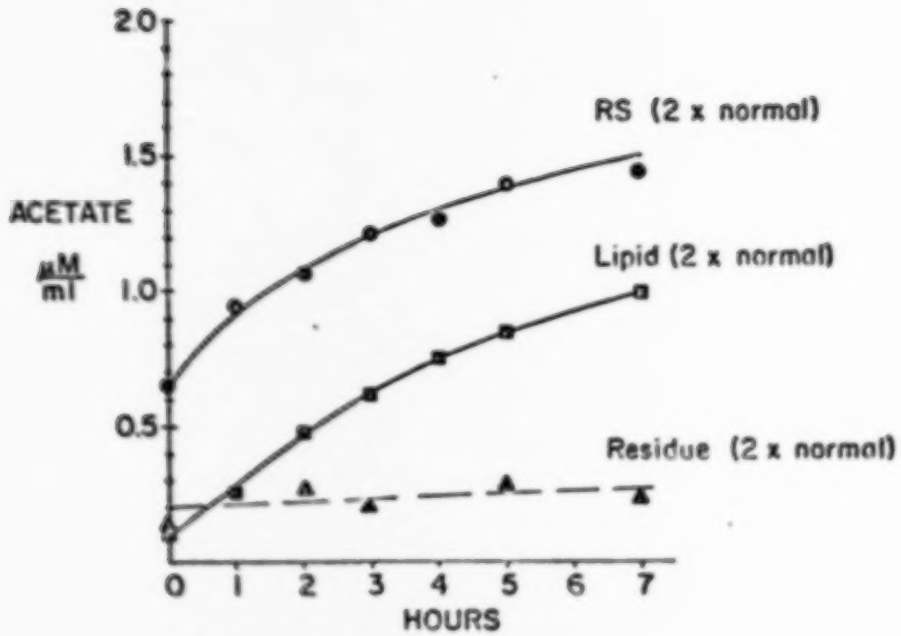
Volatile acids production during the fermentation of raw sludge.

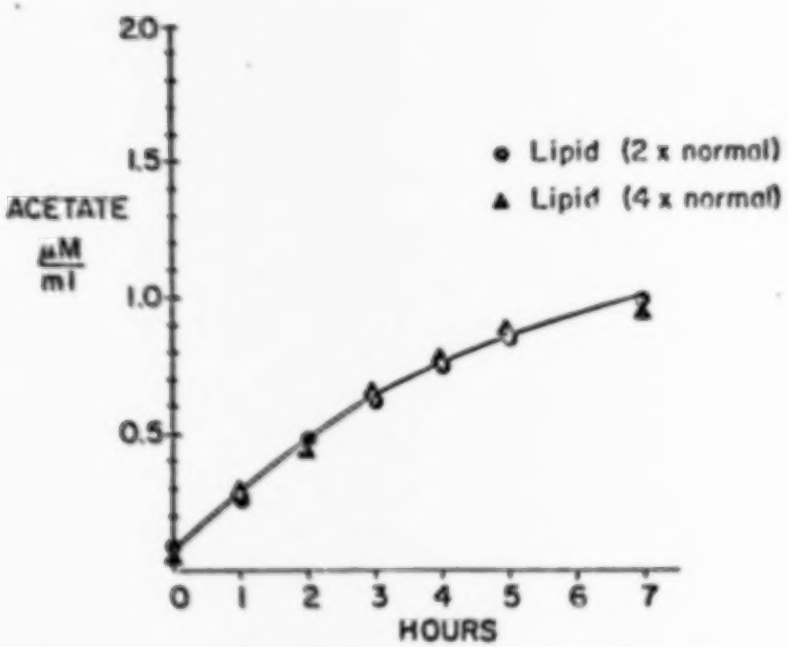
FIGURE 8



Acetate production from the solids and supernatant fractions of raw sludge.

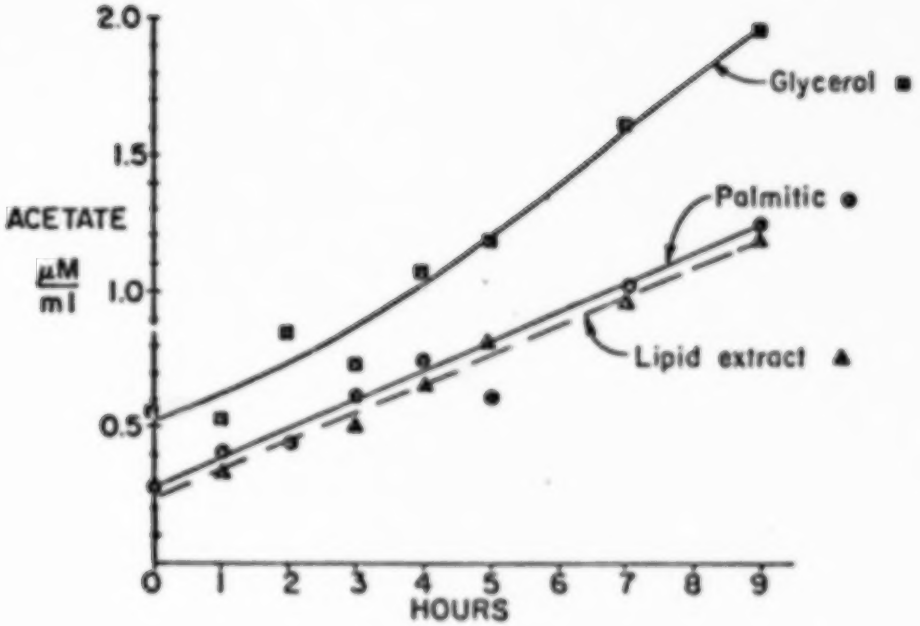
FIDURE 9

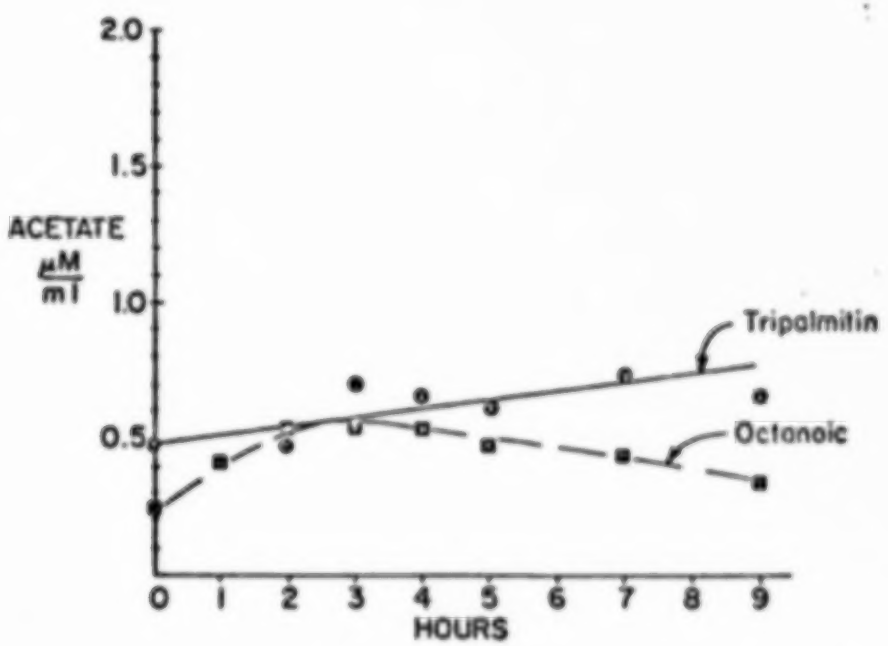




Acetate production from lipid extract of raw sludge.

FIGURE 10





Production of acetate from different lipids.

FIGURE 11

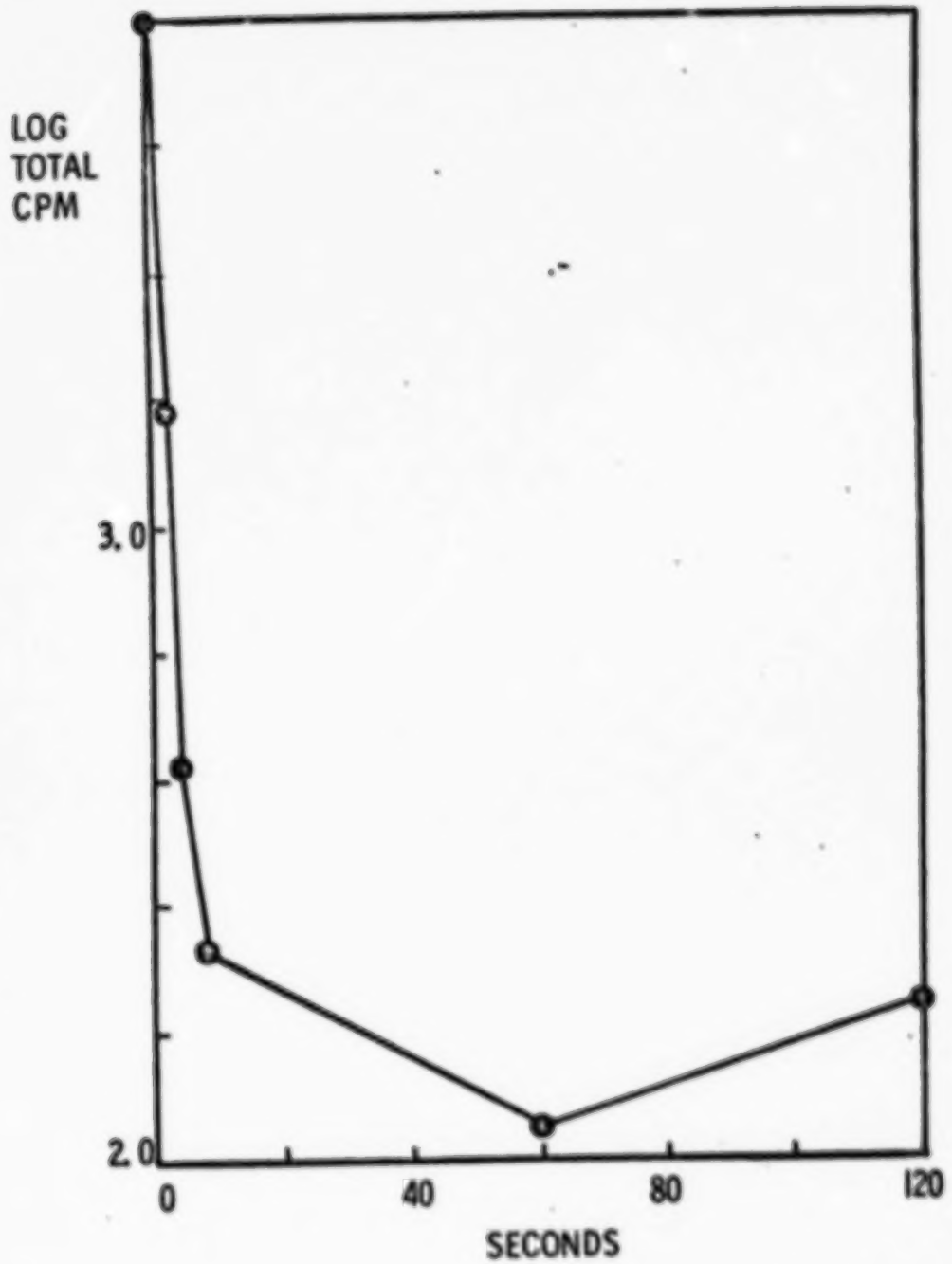


FIGURE 12

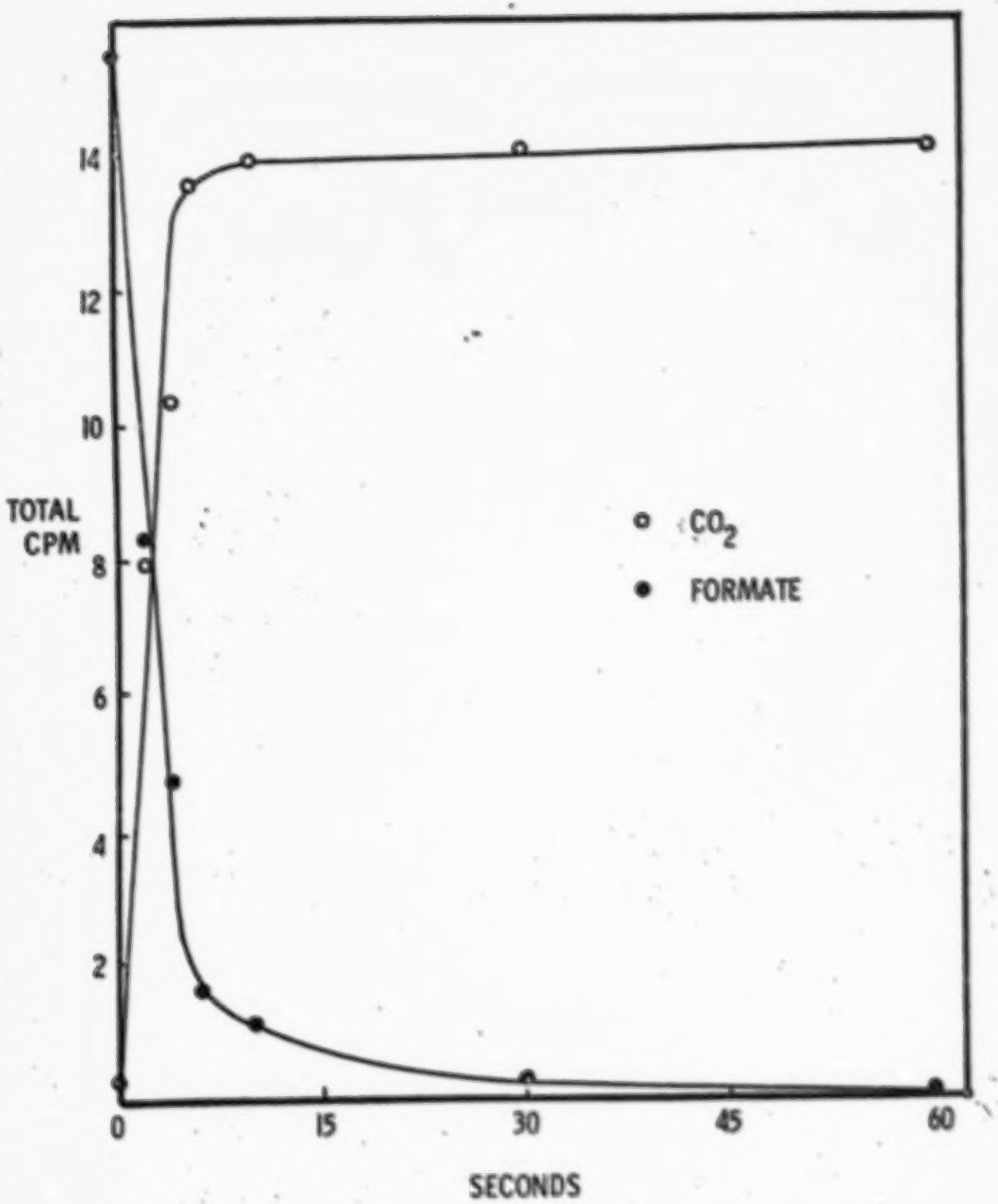


FIGURE 13

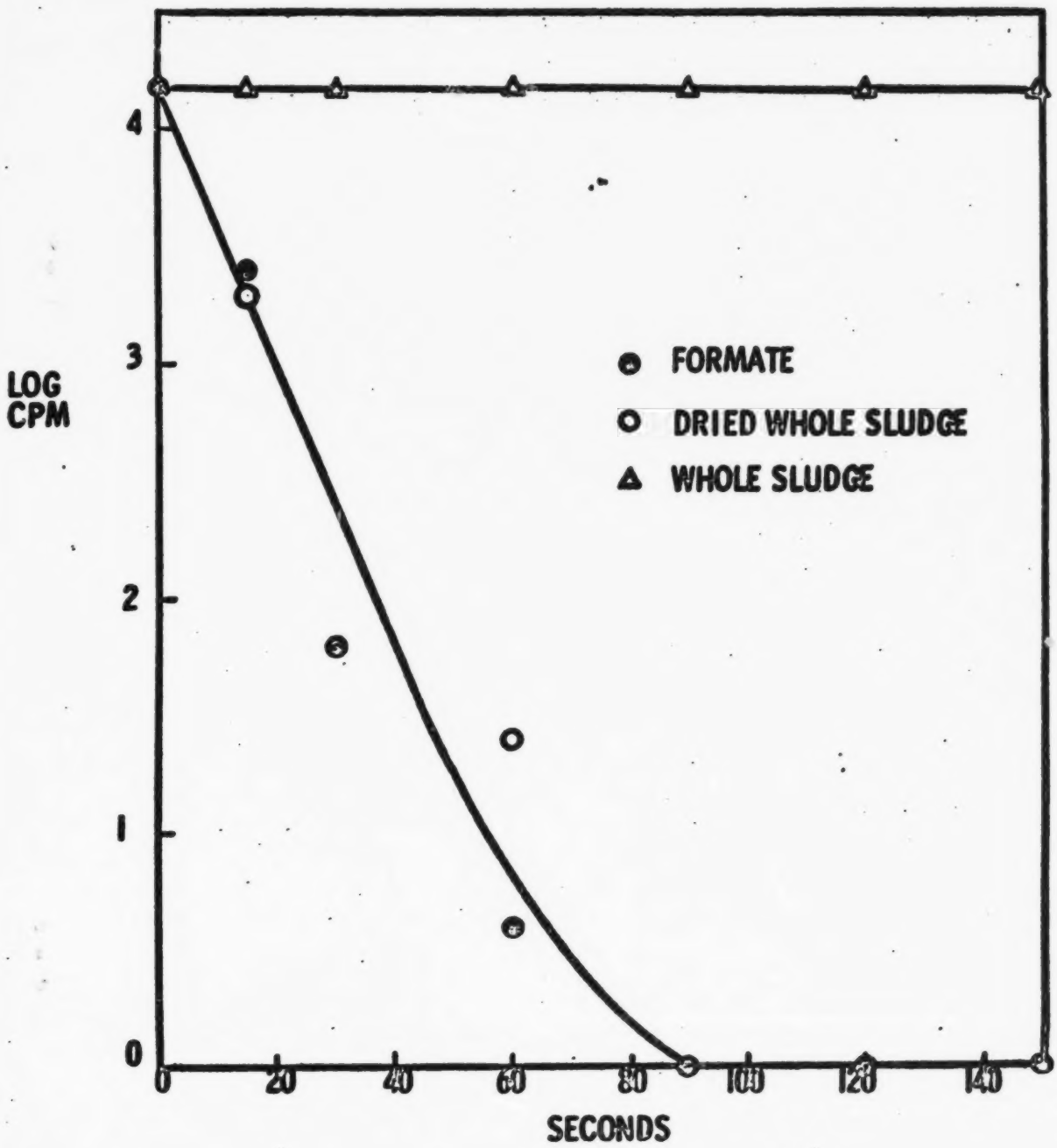

LOG CPM
4
3
2
1
0
0
20
40
60
80
100
120
140
SECONDS
FORMATE
DRIED WHOLE SLUDGE
WHOLE SLUDGE

FIGURE 14

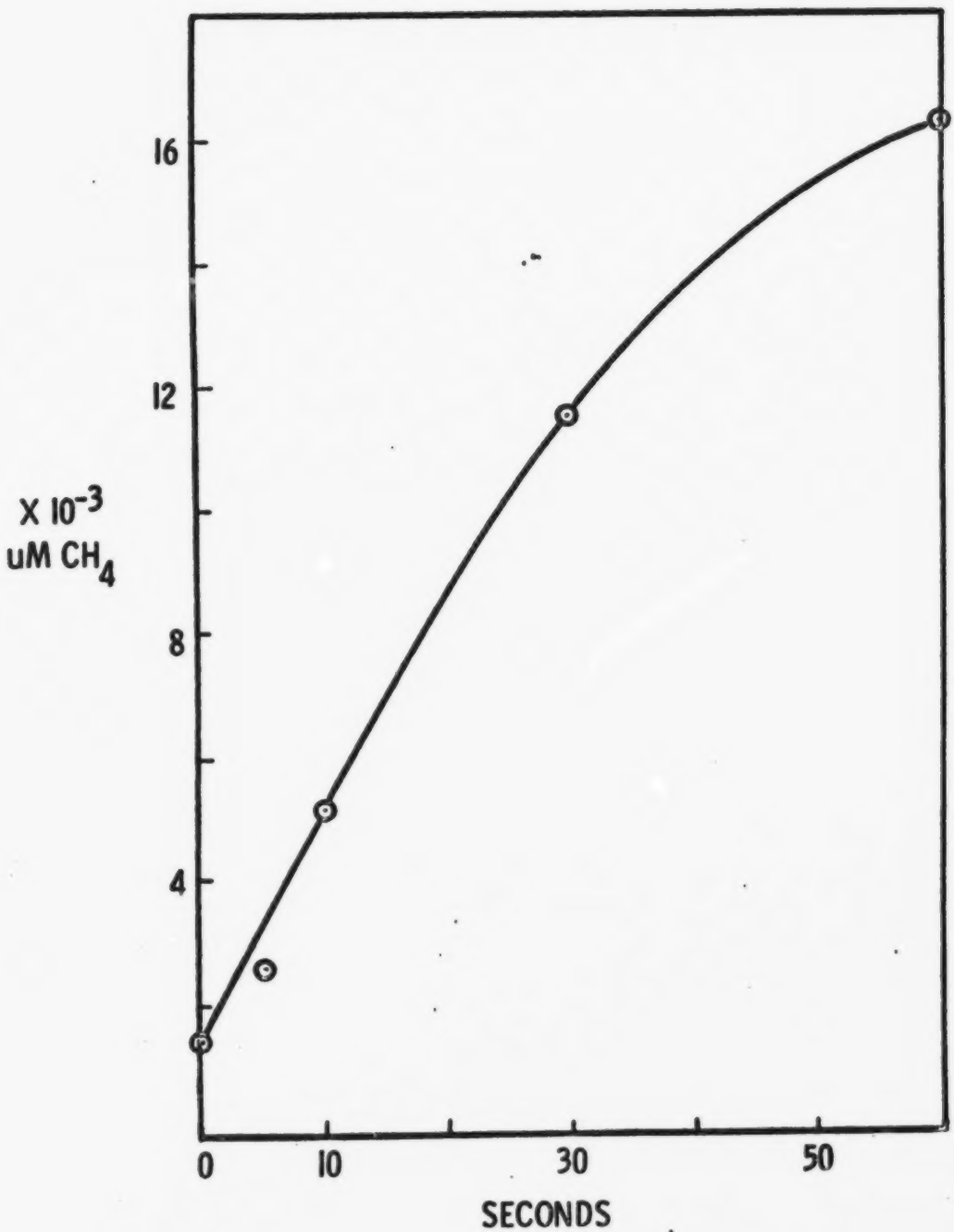


FIGURE 15